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January 19, 1998

Commissioner of Patents and Trademarks
BOX PATENT APPLICATION
Washington, D.C. 20231

Re: New Patent Application
1α-HYDROXYVITAMIN D_5, ITS SYNTHESIS AND USE IN CANCER PREVENTION AND THERAPY
(Priority Application No. 60/039,106 filed 02/25/97)
Inventors: Moriarty, R.M.; Penmasta, R.; Guo, L.; Rao, M.S.; and Mehta, R.G.
Assignee: Steroids, Ltd.

Dear Sir:

Transmitted herewith for filing is the patent application of R.M. Moriarty et al. as above identified. Enclosed are:

1. Specification and Claims;
2. Four sheets of drawings;
3. Declaration and Power of Attorney;
4. Verified Statement (Declaration) Claiming Small Entity Status (37 CFR 1.9(f) & 1.27(b)) -- Independent Inventors;
5. Assignment of Invention and Assignment Cover Sheet;
6. A check in the amount of $435, representing the $395 filing fee for a small entity, calculated as shown below and the $40 Patent Assignment Recordation fee; and
7. A self-addressed stamped return postal card.

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This patent application claims the benefit under 35 USC § 119(e) of the priority date of provisional application Serial No. 60/039,106, filed Feb. 25, 1997.

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Sincerely yours,

Harold J. Fassnacht

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Reg. No. 35,507

Enclosures

cc: Liang Guo w/enclosures
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Application for Letters Patent

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For

1α-HYDROXYVITAMIN D_5, ITS SYNTHESIS AND USE IN CANCER PREVENTION AND THERAPY

Specification

1α-Hydroxyvitamin D_5, ITS SYNTHESIS AND USE IN CANCER PREVENTION AND THERAPY

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/039,106 filed February 25, 1997.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to biologically active vitamin D_5 compounds. More specifically, this invention relates to a series of novel D_5 compounds, including the compound 1α-Hydroxyvitamin D_5, their synthesis and their use in cancer prevention and therapy.

Description of the Related Art

Vitamin D is a secosteroid and is classified as a hormone within the steroid hormone family. Vitamin D's are differentiated on the basis of side-chain chemical structures into different series, e.g., D_2, D_3, D_4, D_5, and D_6. To date, attention has been focused almost exclusively on the vitamin D_3 series of compounds. In its biological form, vitamin D_3 is inactive until it is metabolized to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the natural metabolite. The inactive 24-hydroxy form of the hormone is excreted from the body. The active metabolite 1α,25$(OH)_2D_3$ has been shown to suppress the growth in

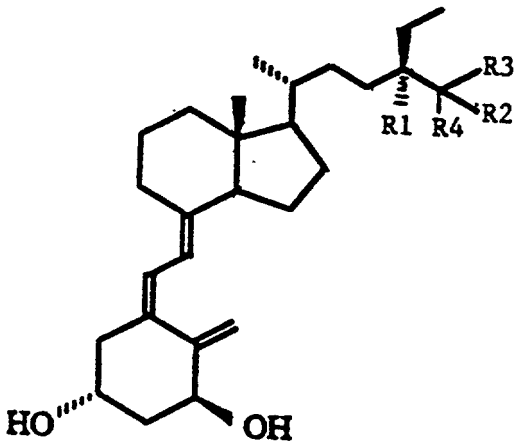
vitro of many neoplastic cells, including breast cancer cells. In addition, treatment of colon cancer cells and leukemia cells with $1\alpha,25(OH)_2D_3$ results in a reduction in the growth rate of these cells.

One of the limiting factors in the successful use of vitamin D_3 in cancer prevention or cancer therapy is its calcemic activity, i.e., the potentially fatal build-up of calcium in the body. The concentrations of vitamin D_3 required to suppress growth of neoplastic cells can cause hypercalcemia and death. Therefore, in recent years, numerous analogues of vitamin D have been synthesized that possess reduced calcemic activity without compromising their antiproliferative activity. The differences in structures of these new compounds arise mostly from modifications in the A and D rings and side chain of the vitamin.

We have synthesized the novel compound 1α-Hydroxyvitamin D_5 [$1\alpha(OH)D_5$] and compared its effectiveness as a chemopreventative to the active metabolite of vitamin D_3. We have also attempted to determine the possible mechanism of such chemopreventative action by studying the expression of vitamin D receptors (VDRs) and transforming growth factor-β (TGF-β) in normal mammary epithelial cells.

SUMMARY OF THE INVENTION

This invention pertains to novel vitamin D_5 compounds including 1α-Hydroxyvitamin D_5, their synthesis, and a method for preventing and treating cancer using these compounds. 1α-Hydroxyvitamin D_5 has the following structure:



where $R_1 = R_4 = H$ and $R_2 = R_3 = CH_3$.

We have synthesized 1α-Hydroxyvitamin D_5 starting with stigmasterol, although sitosterol may also be used as a starting material. Stigmasterol was converted to the 7-dehydro analogue and in turn to the vitamin D_5. The conversion of vitamin D_5 to 1α-Hydroxyvitamin D_5 was accomplished using literature procedures.

1α-Hydroxyvitamin D_5 is a white solid having a molecular formula of $C_{29}H_{48}O_2$ and a molecular weight of 428.7. The 1α-Hydroxyvitamin D_5 was fully characterized by ^1H NMR (400 Mhz), Mass Spectrum [CI], FTIR and UV. Purity was determined by straight and reverse phase high-pressure liquid chromatography (HPLC).

Usefulness of 1α-Hydroxyvitamin D_5: 1α-Hydroxyvitamin D_5 [1α(OH)D_5] is useful because it exhibits pharmacological activity in animals. In particular, preliminary studies in mice indicate 1α-Hydroxyvitamin D_5 is useful in preventing development of carcinogen-induced precancerous lesions at non toxic concentrations.

Use of 1α-Hydroxyvitamin D_5 in Cancer Prevention: Results show that the vitamin D_5 analogue 1α(OH)D_5 inhibits 7,12 dimethylbenz[a]anthracene (DMBA) induced mammary lesions in mammary gland organ culture. This assay has been used to predict possible chemopreventive agents in future clinical trials by the National Cancer Institute. The inhibitions of induction of lesions was accompanied by induction of vitamin D receptors and transforming growth factor β1.

1α-Hydroxyvitamin D_5 is less calcemic than a majority of the analogues of vitamin D_3. This will allow its possible use in prevention of cancer for women at high risk of developing cancer such as women with a family history of cancer or women who may be at a risk of developing disease in the contralateral breast. In addition to breast cancer prevention, the analogue 1α(OH)D_5 may be employed for prevention of cancers of other sites.

Use of 1α-Hydroxyvitamin D_5 in Cancer Therapy: Our studies showed that 1α(OH)D_5 inhibited growth of several human breast cancer cell lines, including ZR 75, T47D, MCF10neo, MCF-7, and BCA-4. The agent differentiates the cells making them less effective for forming cancers. Once the cells were

differentiated with the analogue of D_5, they did not grow in athymic mice when transplanted. Similarly, injection of 8 mg of $1\alpha(OH)D_5$ (3 X week/2 months) to athymic mice bearing breast cancer cells inhibited growth of cancer cells in the animals. These results clearly suggest possible use of analogues of D_5 as chemotherapeutic agents or as adjuvants to chemotherapeutic protocol.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will hereinafter be described in conjunction with the appended drawings.

Figure 1 illustrates the synthesis of 1α-Hydroxyvitamin D_5 from stigmasterol; and

Figure 2 illustrates the various analogues of 1α-Hydroxyvitamin D_5.

DETAILED DESCRIPTION OF THE DRAWINGS

We have synthesized the novel compound 1α-Hydroxyvitamin D_5 and compared its calcemic activity, cancer prevention efficacy and toxicity to that of the active metabolite of vitamin D_3. We have also attempted to determine the possible mechanism of the chemopreventive activity of 1α-Hydroxyvitamin D_5 by studying the expression of VDRs and TGF-β1 in normal mammary epithelial cells.

I. SYNTHESIS OF 1α-Hydroxyvitamin D_5

1α-Hydroxyvitamin D_5 was prepared by the synthesis outlined

in Figure 1 and described in detail below. Numbers in parentheses refer to numerals in Fig. 1.

Step 1 - Preparation of stigmasterol tosylate (2): To a solution of stigmasterol (1) (50 g, 121.15 mmol) in anhydrous pyridine (400 ml) was added tosyl chloride (46.19 g, 242.3 mmol) under argon. The solution was stirred overnight at room temperature (20 hours) in the dark. The reaction mixture was poured into a 400 mL cold 5% $NaHCO_3$ solution. The pale crystalline precipitate was filtered, washed with water and air dried to yield 65g (95%) of stigmasterol tosylate (2).

Step 2 - Preparation of stigmasterol methyl ether (3): A suspension of stigmasterol tosylate (2) (64 g, 112.9 mmol) and potassium acetate (70 g, 713.19 mmol) in anhydrous methanol (1500 mL) was refluxed for 4.5 h under argon atmosphere. The methanol was evaporated in vacuo, and then ether (2 L) was added, washed with water (500 mL), 5% $NaHCO_3$ (2 X 400 mL) and brine (400 mL) and dried ($MgSO_4$). The solvent was evaporated in vacuo to afford 47 g (92%) of stigmasterol methyl ether (3) as a pale yellow viscous liquid.

Step 3 - Preparation of sitosterol methyl ether (4): A solution of stigmasterol methyl ether (3) (10 g, 23.43 mmol) in ethyl acetate (250 mL) and 10% Pd-C (3 g) was shaken in Parr hydrogen apparatus for 4 h (30-40 psi). The Pd-C was filtered through Celite. The solvent was removed *in vacuo* to afford sitosterol methyl ether (4) in quantitative yield.

Step 4 - Preparation of Sitosterol acetate (5): To a

solution of sitosterol methyl ether (4) (50 g, 116.62 mmol) in glacial acetic acid (1 L) was added zinc acetate (65 g, 354.3 mmol). The reaction mixture was refluxed for 6 h, cooled, then 1.5 L of water was added. The resulting white precipitate was filtered, washed with water and air dried. Recrystallization in ether-methanol afforded 42 g (79%) of sitosterol acetate (5) as a white crystalline solid.

Step 5 - Preparation of 7-Dehydrositosterol acetate (6): A suspension of sitosterol acetate (5) (10 g, 21.89 mmol), anhydrous $NaHCO_3$ (9.19 G, 109.45 mmol) and dibromantin in heptane (250 mL) was refluxed for 2 h under argon atmosphere. The reaction mixture was cooled to room temperature and filtered, and then the solvent was removed *in vacuo*. To the reaction flask, THF (50 mL) was added followed by tetrabutylammonium bromide (0.65 g, 2.02 mmol). The solution was stirred at room temperature for 30 minutes under argon atmosphere. To this reaction mixture tetrabutylammonium fluoride (112 mL, 1 M solution in THF) was added and followed by s-collidine (5 mL). Then the reaction mixture was stirred at room temperature for 20 h. The reaction mixture was diluted with ether (1.5 L), then water (600 mL) was added. The crude reaction mixture was transferred to a separating funnel, the water layer was removed, the organic layer was washed with water (500 mL), 1 N HCl (2 X 600 mL), water (600 mL), then brine (500 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to afford a dark brown viscous liquid. The crude reaction mixture was purified by

column chromatography (silica gel, ethyl acetate-hexane 1:9 mixture as eluent) to afford 6.5 g, (75%) 7-dehydrositosterol acetate (6) as a pale brown viscous liquid.

Step 6 - Preparation of 7-Dehydrositosterol (7): To a solution of 7-dehydrositosterol acetate (6) (2.5 g, 5.5 mmol) in dry ether (200 mL) was added lithium aluminum hydride (2.09 g, 55.0 mmol). The reaction mixture was stirred at room temperature for 2 h, then cooled with an ice-water bath and the excess water (5 mL). After 30 minutes, ether (100 mL) was added and filtered. The cake was washed with ether (2 X 100 mL) and the combined organic extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford 7-dehydrositosterol (7) in quantitative yield.

Step 7 - Preparation of Previtamin D_5 (8): 7-Dehydrositosterol (7) (1.5 g, 3.63 mmol) was dissolved in anhydrous ether (630 mL) and benzene (210 mL) and irradiated with stirring under argon in a water cooled quartz immersion well using a Hanovia medium-pressure mercury vapor lamp for 2 h. The reaction mixture was concentrated *in vacuo* to afford the crude previtamin D_5 as a pale brown viscous liquid. The crude reaction mixture was used without purification in the next step.

Step 8 - Preparation of Vitamin D_5 (9): 7-Dehydrositosterol (7) (1.5 g, 3.63 mmol) in ethanol (200 mL) was heated at 60°C for 4 h. The reaction was monitored by TLC. The solution was concentrated *in vacuo* and the crude vitamin D_5 was purified on a silica gel column using 20% ethyl acetate in hexane to yield 600 mg (40%) of Vitamin D_5 (9).

Step 9 - Preparation of Vitamin D_5 tosylate (10): To a solution of Vitamin D_5 (9) (1.6 g, 3.88 mmol) in dry pyridine (20 mL) was added p-toluenesulfonyl chloride (2.22 g, 11.63 mmol). The reaction mixture was stirred under argon for 20 h at room temperature then poured into a cold saturated $NaHCO_3$ solution (100 mL). The mixture was extracted with ether (3 X 200 mL) and the combined organic extracts were washed with 5% HCl (2 X 200 mL), saturated sodium bicarbonate (2 X 200 mL) and brine (200 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield 2 g (98%) of Vitamin D^5 tosylate (10) as a brown viscous liquid.

Step 10 - Preparation of 3,5-Cyclovitamin D_5 (11): To a solution of Vitamin D_5 tosylate (10) (2 g, 3.53 mmol) in anhydrous methanol (250 mL) was added sodium bicarbonate (18 g, 214.26 mmol. The reaction mixture was heated under reflux for 8 h, then cooled and concentrated *in vacuo*. Water (300 mL) was added to the residue and the mixture was extracted with ether (2 X 300 mL). The combined organic extracts were washed with brine, dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield 1.18 g (78%) of 3,5-cyclovitamin D_5 (11) as an oil.

Step 11 - Preparation of 1α-Hydroxy-3,5-Cyclovitamin D_5 (12): To a suspension of selenium dioxide (222 mg. 2 mmol) in dry methylene chloride (160 mL) was added t-butyl hydroperoxide (2.9 mL, 8 mmol, 3 M solution in toluene) under argon. The reaction mixture was stirred under argon at room temperature for 3 h, then dry pyridine (0.3 mL) was added followed by a solution of 3,5-cyclovitamin D_5 (11) (1.5 g, 3.52 mmol) in dry methylene

chloride (50 mL). The reaction mixture was stirred at room temperature for 30 minutes, then 10% NaOH solution (60 mL) was added and the mixture was extracted with ether (3 X 250 mL). The combined organic extracts were washed with 10% NaOH solution (2 X 200 mL), water (2 X 200 mL) and brine (200 mL) and dried ($MgSO_4$) and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography using 20% ethyl acetate in hexane to yield 545 mg (35%) of 1α-hydroxy-3,5-cyclovitamin D_5 (12) as an oil.

Step 12 - Preparation of 1α-Hydroxyvitamin D_5 (13): A solution of 1α-hydroxy-3,5-cyclovitamin D_5 (12) (360 mg, 0.813 mmol) in DMSO (4 mL) and acetic acid (3.5 mL) was stirred and heated at 54-55°C for 1 h under argon. The reaction mixture was poured into crushed ice (100 g), saturated $NaHCO_3$ (80 mL) was added to it, and the mixture was extracted with ether (3 X 150 mL). The combined organic extracts were washed with saturated $NaHCO_3$ solution (2 X 200 mL), water (2 X 150 mL) and brine (200 mL), dried ($MgSO_4$) and concentrated *in vacuo*, to yield 331 mg (95%) of a mixture of 1α-Hydroxyvitamin D_5 (13) and its 5,6-*trans* isomer (14).

Step 13 - Purification of 1α-Hydroxyvitamin D_5 (13): The crude reaction mixture of 1α-Hydroxyvitamin D_5 (13) and its 5,6-*trans* isomer (14) (320 mg, 0.75 mmol) was dissolved in ethyl acetate (70 mL) and then maleic anhydride (73 mg, 0.75 mmol) was added. The reaction mixture was stirred at 35°C for 24 h under argon. The solution was concentrated *in vacuo*. The crude

residue was purified on a silica gel column using 50 % ethyl acetate in hexane to yield 150 mg (47%) of 1α-Hydroxyvitamin D_5 as a white solid. The compound (13) was crystallized from methylformate as white needles and further purified by HPLC (4.6 X 26 cm, C-18 column, CH_3 CN:$H_2$0 9:1) to afford 80 mg of 1α-Hydroxyvitamin D_5 (13), > 99% purity: mp 145-146°C; IR (KBr): 3416 and 1638 cm^{-1}; UV (CH_3OH): λ max $^{265\ nm}$ (ε 18,913); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.54 (S, 3H, 18-CH_3, 0.72-0.98 (m, 9H), 0.92 (d, 3H, J=6Hz, C21-CH_3), 4.24 (m, 1H, 1-H),4.43 (m, 1 H, 3-H), 5.0 (m, sharp, 1H, 19 (E)-H), 5.33 (m, sharp, 1H, 19 (Z)-H), 6.01 (d, 1H, J=11.3 Hz, 7-H), 6.38 (d, 1H, J-11.3 Hz, 6-H); MS (CI) m/e 429 (M^++1, 37%).

Thus the present invention provides the compound 1α-Hydroxyvitamin D_5 indicated by numeral (13) in Figures 1 and 2, and compounds (13a) - (13e) in Figure 2 obtained from 1α-Hydroxyvitamin D_5 using literature procedures. In addition to the above compounds, the invention also provides compounds with stereochemistry at carbon centers C_1 (R or S), C_3 (R or S), C_{20} (R or S) and C_{24} (R or S) in Figure 2.

II. COMPARISON OF CALCEMIC ACTIVITY, CHEMOPREVENTIVE ACTIVITY, AND TOXICITY OF 1α-Hydroxyvitamin D_5 AND 1α,25-DIHYDROVITAMIN D_3

A. Experimental Equipment and Methods

1. High-Pressure Liquid Chromatography (HPLC) Analysis of Vitamin D Analogues

The vitamin D_3 and D_5 analogues were dissolved in acetonitrile at a final concentration of 0.2 mg/mL. Aliquots (10 μL) were injected on a Suplex PKB-100 HPLC column at ambient temperature. The HPLC was carried out with the use of an Hitachi L-6000 pump, an L-4200 UV-VIS detector, and an AS-2000 autosampler (Hitachi Instruments, Inc., Naperville, IL). The vitamin D analogues were eluted with the mobile phase of acetonitrile-methanol-water (52:30:18, vol/vol) with the flow rate at 1 mL/minute, and the elution profile was monitored at 254 nm.

Both 1α-Hydroxyvitamin D_5 and 1α,25-dihydroxyvitamin D_3 analogues exhibited about 98% purity. Stability studies have suggested that both can be stored in powder form for a year at 20°C, whereas in solution they are stable for one month at the same temperature.

2. Measurement Of Calcemic Activity In Rats Administered Vitamin D Analogues

Three-week-old Sprangue-Dawley male rats were obtained from the Holtzman Laboratory, Madison, WI. Up to three rats were housed together in a polycarbonate cage. The animal cages were kept under yellow light. The rats (eight to 10 per group per concentration of both vitamin D analogues used) were fed a

vitamin D-free diet containing 0.47 g/100 g calcium and 0.3 g/100 g phosphorus. After the rats had consumed this diet for 3 weeks, their plasma calcium levels were measured. Rats exhibiting plasma calcium levels of less than 6.0 mg/dL were considered to be vitamin D deficient. Such rats were administered appropriate vitamin D analogues intragastrically for 14 days. At the end of this period, the plasma calcium levels were again measured.

3. Induction Of Preneoplastic Lesions In Mammary Glands And Their Prevention By Vitamin D_3 and D_5 Analogues

Young, virgin BALB/c female mice, 3-4 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA. The mice were pretreated for 9 days with 17β-estradiol (1 μg in 0.1 mL saline per animal) and progesterone (1 mg in 0.1 mL saline per animal). They were then killed by cervical dislocation, and the thoracic pair of mammary glands was dissected out on silk rafts and incubated for 10 days in Waymouth MB752 medium (Life Technologies, Inc. [GIBCO BRL], Gauthersburg, MD) containing the following growth-promoting hormones: insulin (5 μg/mL), prolactin (5 μg/mL), aldosterone (1 μg/mL), and hydrocortisone (1 μg/mL).

The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) at a dose of 2 μg/mL was added to the medium on day 3 for 24 hours to induce mammary lesions. The DMBA-containing medium was then removed, and the mammary glands were incubated for an additional 14 days with medium containing only insulin. This procedure allowed the normal glands to undergo structural regression in which all the normal alveolar structures were disintegrated.

However, the alveolar lesions in the carcinogen-treated glands behaved differently. They had acquired altered hormone responsiveness, and these structures did not regress. These structures were termed "mammary lesions."

The vitamin D analogues (ranging in concentration from 0.01 μM to 10.0 μM) were included in the medium during the first 10 days of the in vitro culture to determine if they lowered the incidence of mammary lesion formation. Throughout the culture period, the glands were maintained at 37°C in an environment of 95% air and 5% CO_2.

At the end of the culture period, the glands were fixed in formalin, stained in alum-carmine solution, and evaluated for the presence or absence of mammary lesions. All hormones and chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.

4. Immunohistochemistry Of VDRs And TGF-β1

Normal mouse mammary glands were dissected and incubated with growth-promoting hormones either alone or in the presence of vitamin D analogues for only 3 days. In this experiment, the glands were not exposed to DMBA (see protocol described in the previous section). Instead, the glands were fixed in buffered formalin, and 5-μm-thick sections were prepared for histopathologic evaluations. The sections were mounted on adhesive-coated slides (Superfast: Fisher Scientific Co., Itasca, IL), dried at 60°C for 1 hour, deparaffinized in xylene,

dehydrated in a series of alcohol, and finally washed with phosphate-buffered saline (PBS).

To block the nonspecific antibody reactions, we treated the tissue sections with 5% dried skim milk for 10 minutes and then incubated them with primary mouse antibody (either against VDR or against TGF-β1, both obtained from BioGenex Laboratories, San Ramon, CA) overnight at 0-4^{0}C. The tissues were rinsed in PBS and incubated with biotinylated rabbit anti-mouse antibody (Dako Corp., Carpenteria, CA.) for 10 minutes; the remaining steps were followed according to the manufacturer-specified protocol; i.e., the reaction was stopped by rinsing the sections with PBS, which was followed by a 10 minute incubation with peroxidase-conjugated streptavidin, three 10-minute rinses with PBS, and a 5-minute incubation in a substrate, 3,3'-diaminobenzidine tetrachloride.

The tissues were counterstained with hematoxylin-eosin, dehydrated through graded series of alcohol and xylene, and finally mounted in Permount (Fisher Scientific Co.). Slides were evaluated for the presence or absence of the VDR or TGF-β1 and for the intensity of staining in the positively strained samples.

5. Statistical analysis

Statistical significance of the results was determined by the chi-squared test. All reported *P* values were obtained from two-sided tests.

B. Experimental Results

1. Calcemic Activity:

One of the primary reasons to synthesize new vitamin D agents is to prepare analogues that have reduced calcemic activity compared with that of $1\alpha,25(OH)_2D_3$, but without compromising the antiproliferative activity. We measured the calcemic activity of both 1α-Hydroxyvitamin D_5 and 1α,25-dihydroxyvitamin D_3.

As shown in Table 1, the vehicle-treated control rats showed a plasma calcium concentration of 5.4 ± 0.28 mg/dL (mean ± standard deviation). When the rats were treated with the vitamin D analogues at a dose of 0.042 μg/kg per day, the following plasma calcium concentrations were observed: 6.0 ± 0.63 mg/dL for $1\alpha(OH)D_5$-treated rats (an 11% increase over that of the vehicle-treated control group; P = .121, i.e., not statistically significant when compared with that of the control group) and 8.1 ± 1.2 mg/dL for $1\alpha,25(OH)_2D_3$-treated rats (a 50% increase over that of the control group; P = .002, i.e., statistically significant difference when compared with that of the control group). At a higher concentration of vitamin D analogues (0.25 μg/kg per day), $1\alpha(OH)D_5$ treatment resulted in a plasma calcium concentration of 7.9 ± 1.5 mg/dL compared with 10.1 ± 1.8 mg/dL for $1\alpha,25(OH)_2D_3$ treatment. Although both analogues at this concentration increased the plasma calcium levels in comparison with those in vehicle-treated control rats, these results showed that $1\alpha(OH)D_5$ has overall lower calcemic effects than

$1\alpha,25(OH)_2D_3$.

$1\alpha,25(OH)_2D_3$ treatment resulted in an 87% increase in the plasma calcium level in rats when compared with the vehicle-treated rats. On the other hand, in animals treated with a higher concentration of $1\alpha(OH)D_5$, there was only a 50% increase in the plasma calcium concentration compared with that in the control animals. These results suggest that $1\alpha(OH)D_5$ is much less calcemic than $1\alpha,25(OH)_2D_3$.

Table 1				
Effects of vitamin D analogues on plasma calcium levels in vitamin D-deficient rats				
Treatment[1]	No. of rats	Dose, μg/kg/day	Plasma calcium, mg/dL[2]	P (two-sided test)
None	8	0.0	5.4 ± 0.28	
$1\alpha(OH)D_5$	8 10	0.042 0.25	6.0 ± 0.63 7.9 ± 1.5	.121 .002
$1\alpha,25(OH)_2D_3$	8 10	0.042 0.25	8.1 ± 1.2 10.1 ± 1.8	.001 <.0001

[1] $1\alpha(OH)D_5$ = 1α-Hydroxyvitamin D_5; $1\alpha,25(OH)_2D_3$ = 1α,25-dihydroxyvitamin D_3.

[2] Values = means +/- standard deviation.

2. Efficacy of Cancer Prevention

Traditionally, the effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland. In organ culture, mammary glands from mice respond to a short stimulation with a carcinogen in the presence of appropriate hormones by developing preneoplastic lesions. When implanted in syngeneic hosts, mammary cells prepared from these lesions give rise to adenocarcinomas. Effective chemopreventive agents (e.g., certain retinoids, selenium, oltipraz, and limonene) inhibit the formation of these lesions. The relative activity of chemopreventitive *in vitro* correlates well with their activity in *in vivo* carcinogenesis experiments. Using this traditional model system, we have evaluated the efficacy of 1α-Hydroxyvitamin D_5 [1α(OH)D_5] in preventing 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary lesion formation in a mouse mammary gland organ culture model.

To evaluate the efficacy of the newly synthesized vitamin D_5 analogue in preventing mammary lesion formation, we incubated 15 mammary glands per group (135 glands in total) from BALB/c mice with appropriate hormones and exposed the glands to DMBA on day 3 for 24 hours (*see* "Experimental Equipment and Methods" section). The mammary glands were incubated for 10 days with the vitamin D analogues in concentrations ranging from 0.01 μM to 10.0 μM. The incidence of mammary lesions was calculated for each group and was reported as the ratio of the number of mammary glands showing lesions to the total number of mammary glands at risk.

Table 2 shows the incidence of mammary lesions in various groups treated with vitamin D analogues. In the vitamin D_5-treated group there was a dose-related decrease in the number of glands exhibiting lesions. In the group treated with vitamin D_3, only two of 14 glands developed lesions at a concentration of 0.01 μM. At higher concentrations of this analogue, no mammary lesions were observed.

We calculated the percent inhibition of formation of lesions in each treatment group by comparing the incidence of lesions between the control group and the treatment group. At a concentration of 10.0 μM, both $1\alpha(OH)D_5$ and $1\alpha,25(OH)_2D_3$ inhibited the formation of mammary lesions by 100%.

At a concentration of 0.01 μM, the vitamin D_3 analogue inhibited mammary alveolar lesion formation by 76%; incubation of glands with concentrations of 0.1 μM and higher showed 100% inhibition. In contrast, the vitamin D_5 analogue inhibited the lesion formation in a dose-dependent manner, reaching 100% inhibition at a concentration of 10.0 μM.

Table 2

Effects of vitamin D analogues on incidence of 7,12 dimethylbenz[a]anthracene-induced lesions in BALB/c mouse mammary glands in organ culture

Concentration (μM)	1α-Hydroxyvitamin D_5			1,25α-dihydroxyvitamin D_3		
	No. of glands with lesions/ total No. of glands treated	% incidence	P (two sided test)	No. of glands with lesions/ total No. of glands treated	% incidence	P (two sided test)
None	9/15	60.0		9/15	60.0	
0.01	6/16	37.5	.21	2/14	14.3	.011
0.1	4/16	25.0	.048	0/15	0.0	.003
1.0	2/14	14.3	.011	0/15	0.0	.003
10.0	0/15	0.0	.003	0/15	0.0	.003

3. Toxicity

To determine the effects of vitamin D analogues on the structural differentiation as well as their toxic effects on mammary glands, we incubated mammary glands with growth-promoting hormones for 3 days either alone or in the presence of 0.1 μM or 1.0 μM vitamin D analogues. The control mammary gland structure was represented by normal alveolar and ductal structures.

1α,25(OH)$_2$D$_3$ at a concentration of 0.1 μM did not show toxicity. Mammary glands displayed normal ductal and alveolar structures. At a concentration of 1.0 μM, vitamin D$_3$ analogue treatment resulted in disintegration of ducts and structural toxicity to the glands.

In contrast, treatment with the vitamin D$_5$ analogue at a concentration of 1.0 μM retained the healthy structural characteristics seen in the untreated glands. In fact, some secretion was obvious in the lumen of the ducts.

In summary, 1α,25(OH)$_2$D$_3$ was toxic to the glands at concentrations of 1.0 μM or higher. Treatment of mammary glands with 1α(OH)D$_5$ did not result in any toxicity to the glands.

4. Mechanism of the Vitamin D Chemopreventive Activity

The mechanism of the vitamin D chemopreventive action is not completely understood. Nuclear vitamin D receptor (VDR) protein binding to 1α,25(OH)$_2$D$_3$ has been identified and is shown to be present in a variety of tissues, including normal mammary glands and mammary tumors, as well as in breast cancer cells. In the

cytosol of target organs or cells, $[^3H]1,25(OH)_2D_3$ binds specifically to receptors with a dissociation constant (K_d) ranging from 1×10^{-10} M to 6×10^{-10} M. An increased nuclear VDR concentration has been found to be associated with an enhanced expression of messenger RNA for vitamin D_3 receptors. The VDR gene has been cloned, and the molecular structure of the receptor protein has been determined. The results have demonstrated that the VDR belongs to the steroid-, thyroid-, and retinoid-receptor superfamily. All of these receptors act as ligand-dependent transcription factors that bind to specific DNA sequences. Two classes of response elements have been identified that are activated either by VDR alone or by heterodimers of VDRs and retinoid X receptor (RXR) alpha.

In recent years, considerable attention has been given to the regulation of cell growth by autocrine antiproliferative factors. Inhibition of cancer cell growth is often related to enhanced production of transforming growth factor-β (TGF-β). TGF-β is further subclassified into the following three isoforms of polypeptides: TGF-β1, TGF-β2, and TGF-β3. These isoforms are present in mammalian cells, including breast cancer cells. The isoforms of TGF-β are regulated differentially by steroid and protein hormones. In one report, a hexafluoro analogue of vitamin D_3, 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), induced expression in HL-60 human leukemia cells of TGF-β1 and its type 2 receptors. These results suggest a possible interaction between the function

of VDR and TGF-β regulation. Induction of TGF-β, however, is often reported only in transformed cells. Although the growth-inhibitory role of TGF-β has been reported in the normal mammary gland, induction of TGF-β in response to chemopreventive agents in this tissue has not been reported previously.

Since the role of chemopreventive agents (including vitamin D_3 and vitamin D_5) on the induction of TGF-β in normal mammary epithelial cells has not been studied, the histologic sections of normal mammary glands treated with either only hormones (insulin, progesterone, aldosterone, and hydrocortisone) or hormones plus vitamin D analogues were processed immunohistochemically to investigate the effects of vitamin D analogues on the induction and localization of VDRs and TGF-β1. VDRs were localized in the nuclei of mammary epithelial cells. There was no selective localization of VDRs in ductal or alveolar cells. Treatment with either 1.0 μM 1α(OH)D_5 or 0.1 μM 1α,25(OH)$_2D_3$ induced expression of VDRs detectable in the nuclei of both ductal and alveolar cells. This induction was dependent on the concentration of the analogue; VDR induction was much less at the lower concentration of the vitamin D_5 analogue. For the vitamin D_3 analogue, intense staining was evident at a lower concentration (0.1 μM). However, at a concentration of 1.0 μM, reduced or absent staining was observed as a result of apparent toxicity.

The effects of the vitamin D analogues on the induction of TGF-β1 were also evaluated. We studied tissue sections from the mammary glands treated with the vitamin D analogues or those from

untreated control glands for the induction of TGF-β1. We found extensive induction of TGF-β1 in the cytoplasm of mammary epithelial cells. Again, the pattern of intensity was comparable to that of induction of VDR. The extent of induction of TGF-β1 after treatment with the vitamin D_5 analogue at a concentration of 1.0 μM was similar to that observed with the vitamin D_3 analogue at a concentration of 0.1 μM. However, at a concentration of 1.0 μM of the vitamin D_3 analogue, TGF-β1 expression was much reduced as a result of toxicity. These results indicate that the vitamin D_5 analogue is considerably less toxic than the vitamin D_3 analogue. Moreover, they indicate that this remarkable induction of TGF-β1 in mammary epithelial cells by the vitamin D_5 analogue may be of importance in cancer chemoprevention.

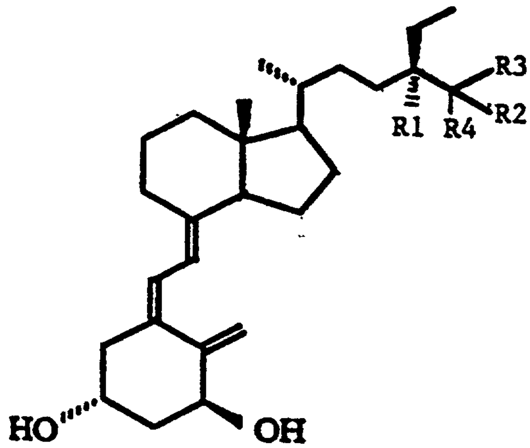
***

Thus we have synthesized a novel vitamin D_5 compound and compared its calcemic activity, cancer prevention efficacy, and toxicity to that of vitamin D_3. We have found that 1α-Hydroxyvitamin D_5, while not completely devoid of calcemic activity, exhibited lower toxicity than 1α,25-dihydroxyvitamin D_3. The present invention represents a first step toward the long-term goal of investigating the efficacy of chemoprevention by and the mechanism(s) of action of analogues of the vitamin D_5 series of compounds. Reduced calcemic activity and lack of toxicity make 1α-Hydroxyvitamin D_5 an attractive candidate for further in vivo chemoprevention studies.

CLAIMS

We claim as our invention:

1. A compound of formula I:



I

wherein:
R1 is hydrogen;
R2 is $-CH_3$;
R3 is $-CH_3$; and
R4 is hydrogen.

2. A compound of formula I wherein:

   a. R1 is hydrogen;
   b. R2 is -OH;
   c. R3 is $-CH_3$; and
   d. R4 is $-CH_3$.

3. A compound of formula I wherein:

   a. R1 is -OH;
   b. R2 is hydrogen;
   c. R3 is $-CH_3$; and
   d. R4 is $-CH_3$.

4. A compound of formula I wherein:

   a. R1 is -OH;
   b. R2 is -OH;
   c. R3 is $-CH_3$; and
   d. R4 is $-CH_3$.

5. A compound of formula I wherein:

a. R1 is hydrogen;
b. R2 is -OH;
c. R3 is $-CF_3$; and
d. R4 is $-CF_3$.

6. A compound of formula I wherein:

a. R1 is hydrogen;
b. R2 is hydrogen;
c. R3 is $-CH_2OH$; and
d. R4 is $-CH_3$.

7. A method of synthesizing the compound of formula I comprising the steps of:

(1) adding tosyl chloride to stigmasterol to make stigmasterol tosylate;

(2) refluxing the stigmasterol tosylate with potassium acetate in methanol to prepare stigmasterol methyl ether;

(3) shaking the stigmasterol methyl ether in ethyl acetate and Pd-C to make sitosterol methyl ether;

(4) refluxing zinc acetate added to a solution of sitosterol methyl ether in acetic acid to make sitosterol acetate;

(5) refluxing a suspension of sitosterol acetate, anhydrous $NaHCO_3$ and dibromantin in heptane; adding THF and tetrabutyl ammonium bromide and tetrabutyl ammonium fluoride and s-collidine to make 7-dehydrositosterol acetate;

(6) adding lithium aluminum hydride to the 7-dehydrositosterol to make 7-dehydrositosterol;

(7) dissolving the 7-dehydrositosterol in anhydrous

ether and benzene and irradiating to make previtamin D_5;

(8) heating a solution of previtamin D_5 in ethanol to make crude vitamin D_5;

(9) adding p-toluene sulfonyl chloride to a solution of vitamin D_5 in pyridine to make vitamin D_5 tosylate;

(10) adding sodium bicarbonate to a solution to a solution of vitamin D_5 tosylate in methanol to make 3,5 cyclovitamin D_5;

(11) adding t-butyl hydroperoxide to a suspension of selenium dioxide in dry methylene chloride and adding a solution of 3,5 cyclovitamin D_5 in dry methylene chloride to make 1α-Hydroxyvitamin-3,5 cyclovitamin D_5;

(12) stirring and heating a solution of 1α-hydroxy 3,5-cyclovitamin D_5 in DMSO and acetic acid to make a mixture of 1α-Hydroxyvitamin D_5 and its 5,6-trans isomer; and

(13) dissolving the mixture of 1α-Hydroxyvitamin D_5 and its 5,6-trans isomer in ethyl acetate and then maleic anhydride, purifying and crystallizing to make 1α-Hydroxyvitamin D_5.

8. A method of preventing the development of carcinogen-induced precancerous lesions which comprises

administering a therapeutically effective amount of the compound of claim 1 to an individual at risk of developing cancer.

9. A method of treating cancer which comprises administering a therapeutically effective amount of the compound of claim 1 to an individual in need of such treatment.

10. The compound of claim 2 with R or S stereochemistry at carbon centers C_1, C_3, C_{20} and C_{24}.

11. The compound of claim 3 with R or S stereochemistry at carbon centers C_1, C_3, C_{20} and C_{24}.

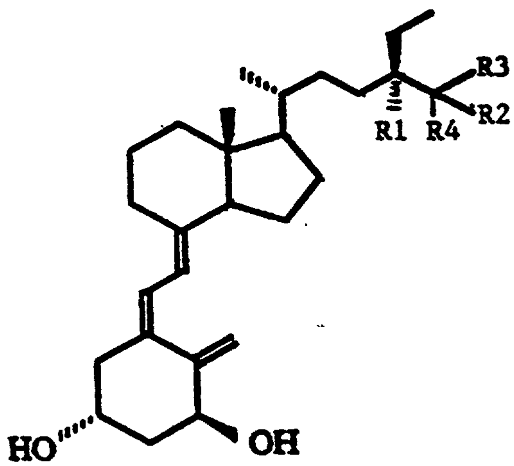
12. The compound of claim 4 with R or S stereochemistry at carbon centers C_1, C_3, C_{20} and C_{24}.

13. The compound of claim 5 with R or S stereochemistry at carbon centers C_1, C_3, C_{20} and C_{24}.

14. The compound of claim 6 with R or S stereochemistry at carbon centers C_1, C_3, C_{20} and C_{24}.

ABSTRACT OF THE DISCLOSURE

A compound of formula I:



I

wherein R1 is hydrogen, R2 is $-CH_3$, R3 is $-CH_3$, and R4 is hydrogen, useful in cancer prevention and therapy.

Preparation of 1-α-hydroxy vitamin D5 from Stigmasterol

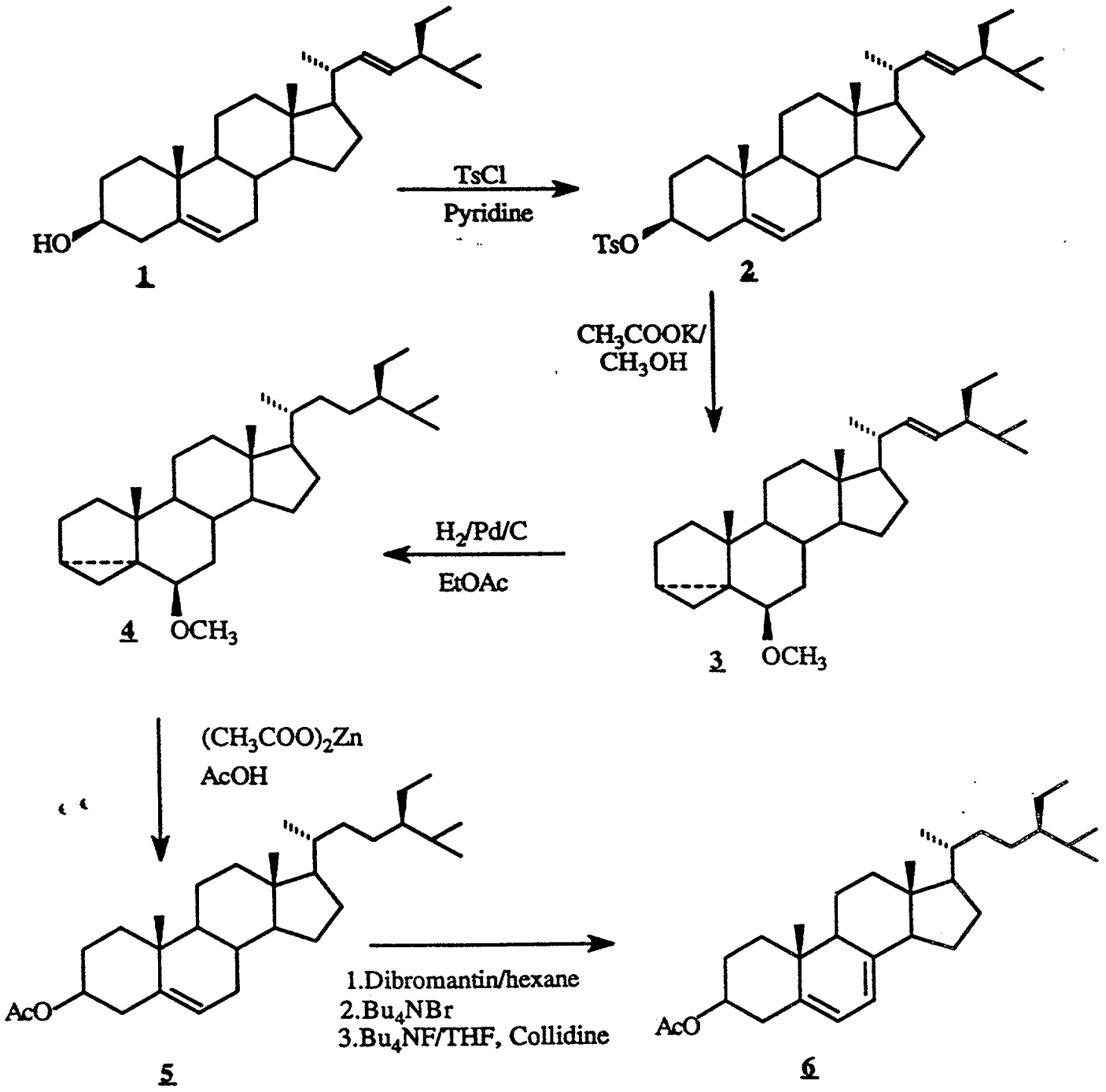


Figure 1

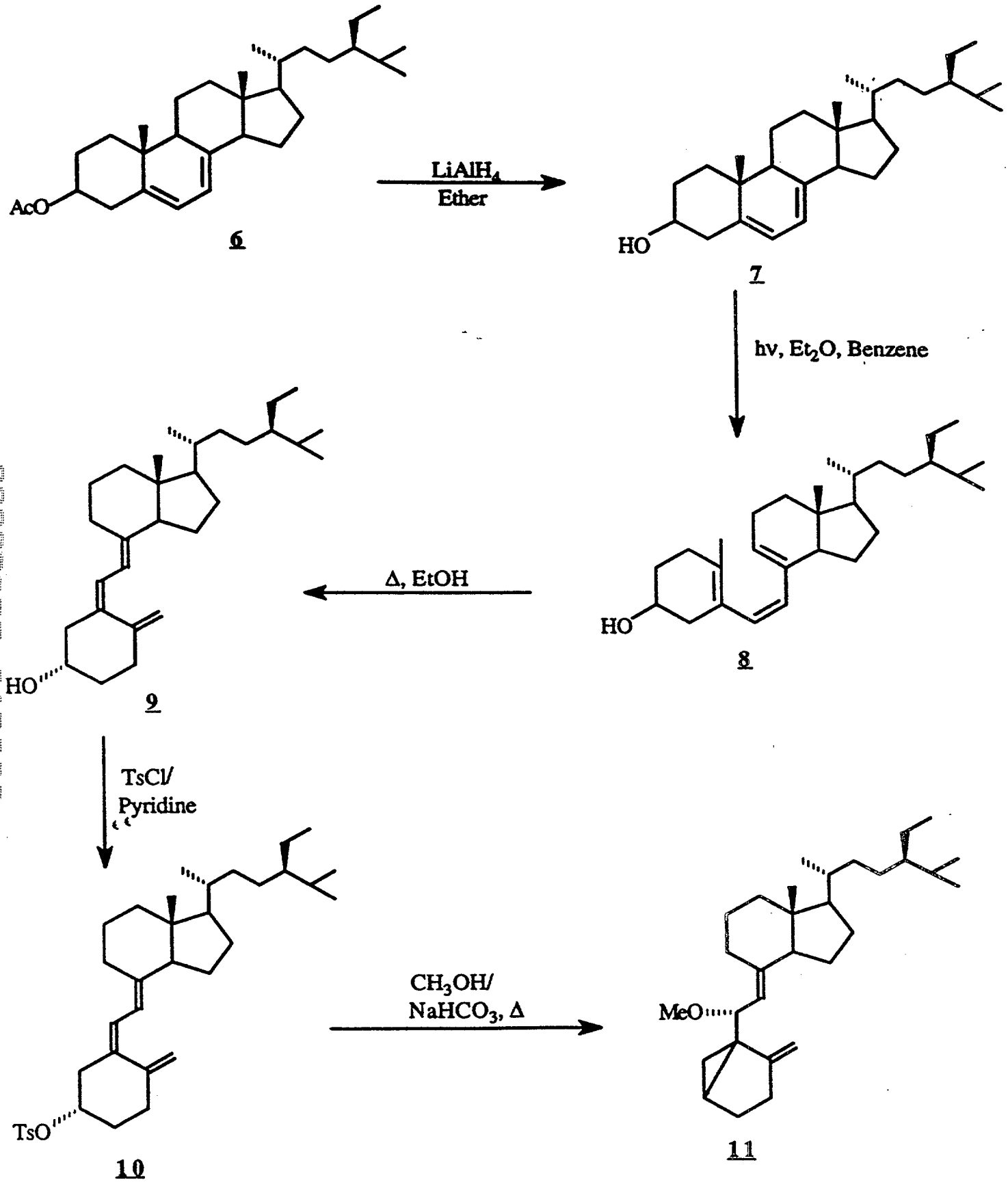


Figure 1 (cont.)

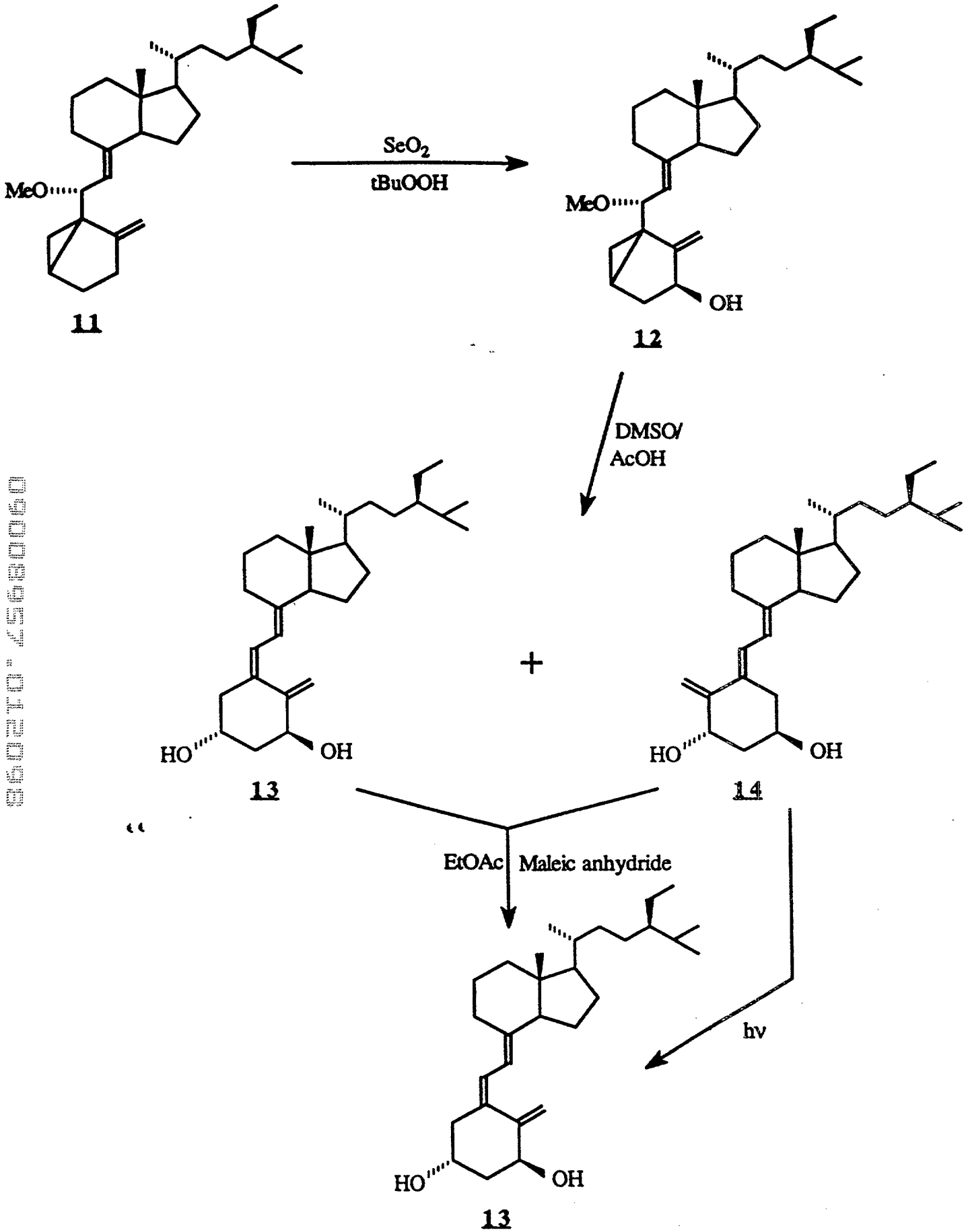
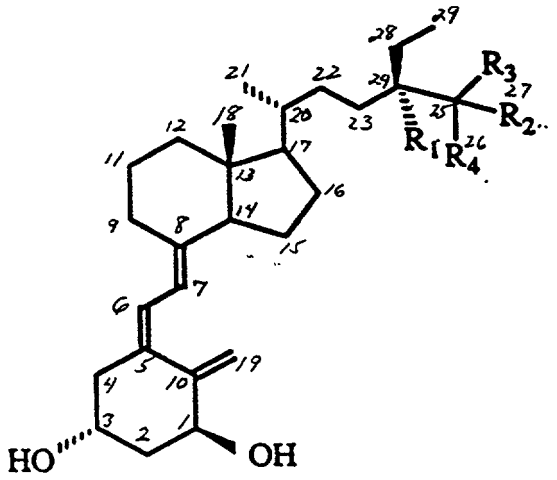


Figure 1 (cont.)

Figure 2



13

13: R1=H; R2=CH3; R3=CH3; R4=H

13a: R_1= H: R_2 = OH; R_3 = R_4 = CH_3

13b: R_1= OH: R_2 = H; R_3 = R_4 = CH_3

13c: R_1= OH: R_2 = OH; R_3 = R_4 = CH_3

13d: R_1= H: R_2 = OH; R_3 = R_4 = CF_3

13e: R_1= H: R_2 = H; R_3 = CH_2OH; R_4 = CH_3

DECLARATION AND POWER OF ATTORNEY

DECLARATION

As the below-named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated next to our names.

We believe we are the original, first and only inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

1α-Hydroxyvitamin D_5, Its Synthesis and Use in Cancer Prevention and Therapy

the specification of which is attached hereto.

We hereby state that we have reviewed and understand the content of the above-identified specification, including the claims and any amendments thereto.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, section 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, section 119, of any foreign applications for patent or inventor's certificate listed below, and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Applications	Priority Claimed
(none)	

We hereby claim the benefit under Title 35, United States Code, section 120 of any United States application listed below, and insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, section 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulation, section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

App. Serial No.	**Filing Date**	**Status**
60/039,106	**02/25/97**	**Provisional**

POWER OF ATTORNEY

As named inventors, we hereby appoint the following attorneys to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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Post Office address: Same as above.

DECLARATION: We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

(Signature of Inventor)

1/11/98
(Date)

(Signature of Inventor)

1/10/98
(Date)

(Signature of Inventor)

1/12/98
(Date)

(Signature of Inventor)

1/12/98
(Date)

(Signature of Inventor)

1/12/98
(Date)

Patent

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION OF:
Robert M. Moriarty
Raju A. Penmasta
Liang Guo
Munagala S. Rao
Rajendra G. Mehta

SERIAL NO.:

FILED:

FOR: 1α-HYDROXYVITAMIN D_5, ITS SYNTHESIS AND USE IN CANCER PREVENTION AND THERAPY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(b)) -- INDEPENDENT INVENTORS

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

As below named inventors, we hereby declare that we qualify as independent inventors as defined in 37 C.F.R. Section 1.9(c) for the purposes of paying reduced fees to the Patent and Trademark Office regarding the invention titled 1α-HYDROXYVITAMIN D_5, ITS SYNTHESIS AND USE IN CANCER PREVENTION AND THERAPY described in the specification filed herewith.

We have not assigned, granted, conveyed or licensed and are under no obligation under contract law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 C.F.R. Section 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 C.F.R. Section 1.9(d) or a nonprofit organization under 37 C.F.R. Section 1.9(e).

Each person, concern, or organization to which we have assigned, granted, conveyed or licensed or are under obligation under contract or law to assign, grant, convey or license any rights in the invention is listed below:

Steroids, Ltd.

We acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of

entitlement to small entity status prior to paying or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Inventor: Robert M. Moriarty

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Date: 1/11/98

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Name of Inventor: Liang Guo

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Name of Inventor: Rajendra G. Mehta

Signature of Inventor: R G Mehta

Date: 1/12/98